Eurosurveillance
Europe's journal on infectious disease epidemiology, prevention and control

Impact factor 5·9

Vol. 21 | Weekly issue 39 | 29 September 2016

Rapid communications

Surveillance report

Letters

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Increase in ECHOvirus 6 infections associated with neurological symptoms in the Netherlands, June to August 2016

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Citation style for this article:
Benschop KS, Geeraedts F, Beuvink B, Spit SA, Fanoy EB, Claas EC, Pas SD, Schuurman R, Verweij JJ, Bruisten SM, Wolthers KC, Niesters HG, Koopmans M, Duizer E, on behalf of VIRO-TypeNed. Increase in ECHOvirus 6 infections associated with neurological symptoms in the Netherlands, June to August 2016. Euro Surveill. 2016;21(39):pii=30351. DOI: http://dx.doi.org/10.2807/1560-7917.ES.2016.21.39.30351

Article submitted on 09 September 2016 / accepted on 29 September 2016 / published on 29 September 2016

The Dutch virus-typing network VIRO-TypeNed reported an increase in ECHOvirus 6 (E-6) infections with neurological symptoms in the Netherlands between June and August 2016. Of the 31 cases detected from January through August 2016, 15 presented with neurological symptoms. Ten of 15 neurological cases were detected in the same province and the identified viruses were genetically related. This report is to alert medical and public health professionals of the circulation of E-6 associated with neurological symptoms.

From June 2016 onwards, an increase in the number of ECHOvirus 6 (E-6) infections was noted by the Dutch virus-typing network VIRO-TypeNed [1]. Among a total of 31 cases, 15 presented with neurological symptoms. Compared with the annual average of four cases with a neurological E-6 infection in the past five years, this increase was statistically significant. Here we aim to alert medical and public health professionals of the increase and circulation of E-6 associated with neurological symptoms.

Epidemiological investigation

In the period from January to August 2016, 242 enterovirus (EV) cases were reported by VIRO-TypeNed [1]. E-6 was the most frequently identified type and accounted for 13% (n = 31) of the EV cases; in previous years, this type had only been detected in on average 4% of the cases, ranging from 0.3% (1/308) in 2010 to 6% (26/464) in 2015. The female:male ratio among E-6 cases was 1:1 and 18 of the 31 cases were younger than seven years (range: 2 weeks–44 years).

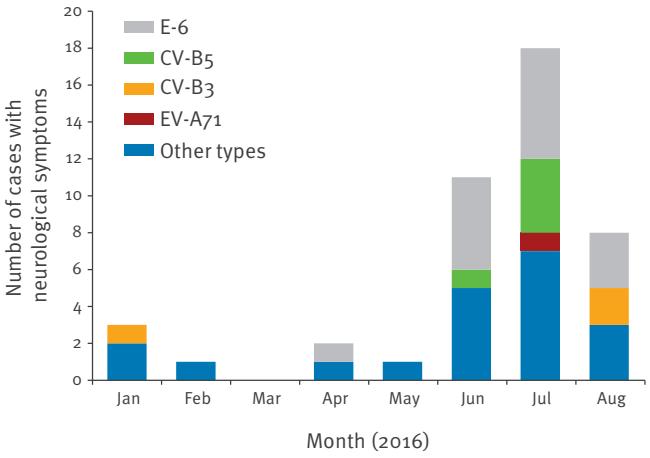
44 (18%) of the total 242 EV cases presented with neurological symptoms and 15 of them were infected with E-6 (Figure 1, Table). Cases that presented with neurological symptoms were defined as patients with aseptic meningitis (n = 4), suspected or undefined neurological presentation (n = 2) or an (unreported) clinical presentation that prompted the physician to examine the cerebrospinal fluid (CSF) (n = 9).

The total number of cases (irrespective of EV type) presenting with neurological symptoms was not increased compared with the respective period (January through August) in the previous five years: the average in this period was 41 cases, ranging from 24 in 2013 to 50 in 2014. However, in 2016, the proportion of E-6 cases with neurological symptoms was significantly higher compared with other EV types with neurological symptoms (p value < 0.05 based on the univariable chi-squared test) (Table). Specifically, the number of cases with neurological symptoms was not increased for any of the other top five detected types (Coxsackievirus (CV)-B3, CV-B5, EV-A71 and CV-A6) (Table).

The 31 E-6 cases detected since January 2016 were identified across the Netherlands. However, from

Figure 1

Monthly distribution of neurological cases with enterovirus infection, reported by VIRO-TypeNed, the Netherlands, January–August 2016 (n = 44)



E-6, CV-B3, CV-B5 and EV-A71 are shown separately as they were among the top five detected types with neurological cases. 'Other types' include CV-A9, CV-B1, CV-B2, CV-B4, E-5, E-7, E-9, E-13, E-18 and E-30 (Table).

June through August 2016, when EV infections associated with neurological symptoms peaked, a cluster of 10 cases infected with the same E-6 strain were found to reside in the same province (6 female and 4 male cases, median age: 27 years; range: 2 weeks–45 years). Nine of those cases presented with neurological symptoms and included seven adults (median: 28 years; range: 27–45). Five of the 10 cases resided in the same municipality and four of them were neurological cases. These five cases included a two-week-old neonate and its mother. The child had a fever, without evident neurological symptoms. Further investigations are being conducted on the clinical and epidemiological characteristics of the cases in the cluster to investigate a possible source and link. In the same province, 36 EV cases were detected in total and clinical information was available for all; of the 26 cases infected with strains other than E-6, only six presented with neurological symptoms; they were infected with CV-B5 (n = 2), CV-B3 (n = 1), E-7 (n = 1), E-18 (n = 1) and E-30 (n = 1).

Phylogenetic investigation

In the phylogenetic analysis based on the partial VP1 region [2], the E-6 strains circulating in the Netherlands since 2010 could be grouped in the previously assigned genogroups B (one strain from 2015), C1 (75 strains from 2011–16), C4 (two strains from 2014) and C9 (65 strains from 2010–16) [3,4]. Twenty-eight of the 31 E-6 strains recovered in 2016, including those from the provincial cluster, could be characterised as C1 (Figure 2), and the three remaining strains as C9. The strains from the cluster were 99.9% homologous.

Outbreak detection and response

EV infection is not notifiable in the Netherlands, but more than 300 EV per year are typed in the poliovirus exclusion and EV surveillance programme. Here, we detected the outbreak in a timely manner through our regular analysis of data in the VIRO-TypeNed database, observing 10 cases in one province that clustered in time and place and had identical molecular types. The cluster was reported to the national early warning committee and surveillance unit of the national institute for public health and the environment (RIVM) in order to alert and create awareness among medical and public health professionals.

Background

E-6 is one of the five most frequently detected EV types associated with neurological symptoms worldwide, next to EV-A71, E-11, E-30 and CV-B5, each accounting for 15–20% of EV types identified in a year [5-10]. EVs are ubiquitous and circulate all year round with peaks in the summer months. Neurological symptoms can be linked to various types. However, there have been years where a majority of neurological cases could be linked to a specific type ([5-10] and data not shown), as is currently seen for E-6 in 2016. E-6 is endemic in the Netherlands and was detected more frequently in 2000 and 2009 by the clinical surveillance as described by van der Sanden et al. (Figure 3) [11].

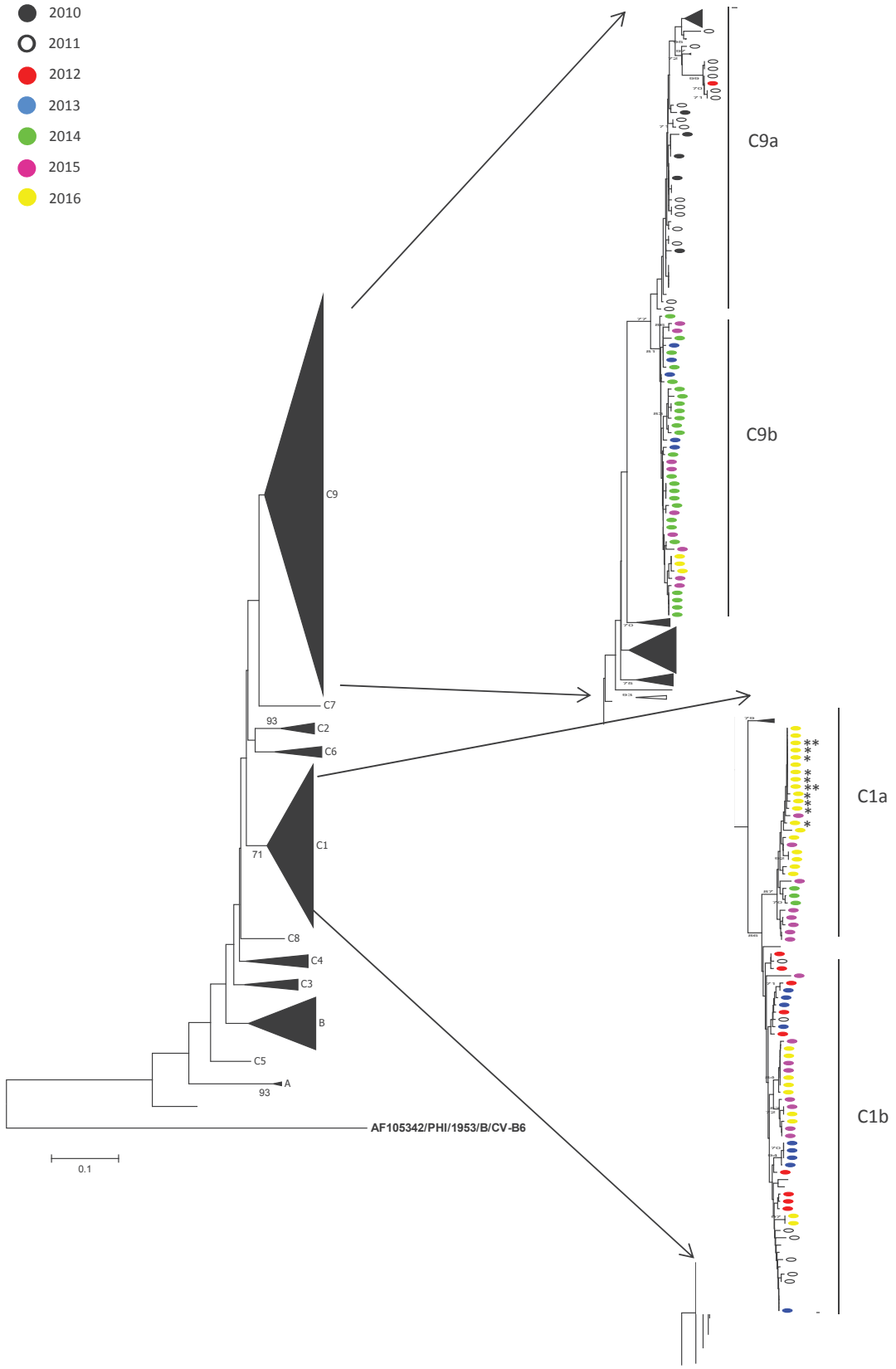
Typing of positive isolates from clinical surveillance is primarily performed to exclude the circulation of poliovirus [11]. The data on non-Polio EV (NPEV) types are used to monitor NPEV circulation and trends in NPEV illness. Since 2010, these data have been collected in a standardised manner through VIRO-TypeNed. VIRO-TypeNed is a virus-typing network using a joint data-sharing database for clinical and public health laboratories to provide a more complete surveillance of EV including genetic, epidemiological, patient and clinical data such as information on gastrointestinal, respiratory and neurological symptoms [1]. Details on the surveillance system (sampling method, detection and typing methods, reporting of data) have been described elsewhere [12]. In short, all EV-positive cases detected through real-time PCR that can be characterised by partial typing of the VP1 region [2] include a minimum dataset including age and sex of patient, type of sample from which the virus was detected, whether the patient was hospitalised, travel history (by country visited) and clinical symptoms in broad categories (skin, neurological, respiratory, enteric). Cases presenting with neurological symptoms are defined as having aseptic meningitis, suspected or undefined neurological presentation, encephalitis, convulsions, or from whom clinical presentation (unreported) was such as to prompt the physician to examine the CSF.

Discussion

On 8 August, the European Centre for Disease Prevention and Control (ECDC) published a rapid risk assessment (RRA) regarding EV detections in severe

Figure 2

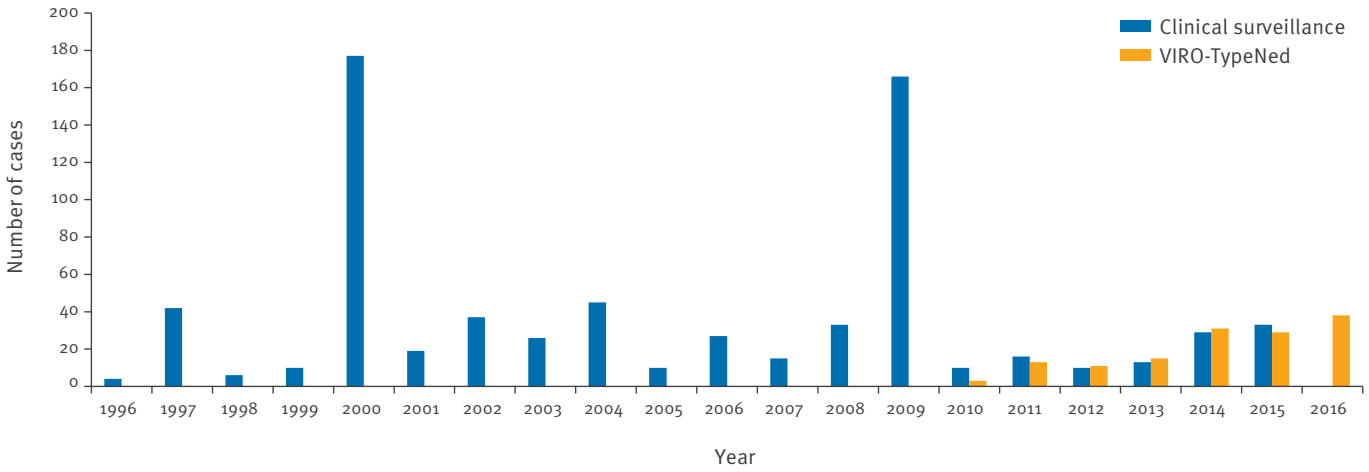
Neighbour-joining (maximum composite likelihood) tree based on a 250 nt VP1 fragment [2] of ECHOvirus 6 from patients, the Netherlands, 2010–2016 (n = 31)



At the nodes, the percentage bootstrap support for branching events after 1,000 replicates are shown. Sequences were aligned using Simmonics sequence editor and the tree was constructed using MEGA6. The years of isolation are depicted in colour-coded circles. The 10 cases from the provincial cluster are indicated by an asterisk and the mother and child by a double asterisk.

Figure 3

Distribution of cases of ECHOvirus 6 infection, the Netherlands, 1996–2015 (clinical surveillance; n = 728) and 2010–2016 (VIRO-TypeNed; n = 133)



Clinical surveillance data are collected once a year, therefore data from 2016 is not available.

neurological cases among children and adults in Europe, to reinforce vigilance for EV-associated neurological disease [13]. Several types were reported across Europe, and alerts were released on an increase in the number of EV-A71 (a new C1 variant) associated with neurological symptoms [13] as well as on an increase of EV-D68 associated with severe respiratory disease.

The E-6 increase represents the second EV signal in the Netherlands in 2016, following the EV-D68 increase seen in the summer of 2016 [13]. Both outbreaks were rapidly detected by the national VIRO-TypeNed surveillance system [1]. As this system includes epidemiological, clinical and molecular typing data, near real-time cluster detection is feasible. At the moment, outbreak detection is based on weekly analysis of the data in the VIRO-TypeNed database. As the majority of EV infections are asymptomatic and mild, not all infections are reported. Therefore, we cannot exclude that the increase is biased based on active reports of severe (neurological) cases. However, a comparison of the data with only severe (neurological) cases in previous years revealed a clear increase for E-6; A majority of these cases were defined based on unreported clinical presentation where CSF was examined. The data on specimen type (CSF) is unbiased and completeness of this dataset is more than 90%.

Phylogenetic analysis suggested that the 2016 outbreak is associated with the C1 strain. Previous reports on E-6 outbreaks were related to C9, for example a major outbreak associated with neurological symptoms in Spain in 2008 [5] and in other countries since 2000 [3]. Analysis of full-length VP1 or full-length genomes as well as serological studies are needed to further investigate the underlying genetical and immunological factors that are responsible for the possible increase in neurological virulence and/or possible increase in circulation (e.g. viral fitness, transmissibility or lack of immunity). Of interest is that while more than half of the E-6 cases seen nationwide occurred in children, most cases belonging to the cluster were older than 27 years, suggesting that severity is not related to a younger age but rather to waning immunity or lack of immunity to the C1 strain.

Preventive measures for EV outbreaks are limited to advice on more stringent cough and hand hygiene or case isolation to prevent nosocomial spread. Treatment options for (severe) EV infections are limited. Based on the humoral responsiveness of EV infection, intravenous IG (IVIG) can be given in severe cases. However, IVIG is not always effective [14]. While antiviral drugs against EV infections are under development, there is still no EV-specific treatment available [15,16]. Several studies have described the use of the capsid inhibitor pleconaril on a compassionate use basis in neonates and immunocompromised patients with severe EV infections, with variable outcome [14]. Other options are drugs marketed for other viral infections or clinical conditions that can be used off-label, however, they have never been clinically tested in EV cases and public health implications are unknown [15].

Table

Enterovirus types reported to VIRO-TypeNed with cases presenting with neurological symptoms, the Netherlands, January–August 2016 (n = 172[a])

EV species	EV type	Number of cases	Number of cases with neurological symptoms
EV-A	EV-A71[b]	20 (13 C2; 7 C1)	1
EV-B	CV-A9	12	3
EV-B	CV-B1	13	2
EV-B	CV-B2	6	1
EV-B	CV-B3[b]	20	3
EV-B	CV-B4	12	2
EV-B	CV-B5[b]	21	5
EV-B	E-5	3	1
EV-B	E-6[b]	31	15
EV-B	E-7	6	1
EV-B	E-9	4	1
EV-B	E-13	10	4
EV-B	E-18	2	1
EV-B	E-30	12	4

CV: Coxsackievirus; E: ECHOvirus; EV: Enterovirus.

[a] For types CV-A2 (n = 1), CV-A5 (n = 1), CV-A6[b] (n = 21), CV-A8 (n = 2), CV-A10 (n = 8), CV-A13 (n = 1), CV-A14 (n = 1), CV-A16 (n = 2), CV-A22 (n = 1), E-3 (n = 2), E-11 (n = 10), E-16 (n = 1), E-25 (n = 2) and EV-D68 (n = 17), no cases presenting with neurological symptoms were reported.

[b] EV types ranked top five detected types (January to August 2016).

Case definition of neurological cases: patients with aseptic meningitis, suspected or undefined neurological presentation, encephalitis, convulsions, or with a clinical presentation (not reported) that prompted the physician to examine the cerebrospinal fluid.

Acknowledgements

We want to thank all the virologists participating in the Weekly Sentinel Surveillance System of the Dutch Working Group for Clinical Virology for collecting and providing positive samples for PV exclusion and NPEV typing. We would also like to the laboratory staff of Laboratories participating in the VIRO-TypeNed project: Edin Jusic, Bas van der Veer, Annemarie van den Brandt and Jeroen Cremer (RIVM); Jolanda Kreeft-Voermans and Mark Verbeek (Erasmus MC); Lisette Rusman (LUMC); Hèlen van Raak and Judith Beuving (St Elisabeth Hospital); Gerrit Koen, Hetty van Eijk, Xiomara Thomas, Rene Minnaar, Karen Dijkamn-deHaan and Sjoerd Rebers (AMC); Randy Poelman, Coretta Van Leer-Buter and Renze Borger (UMCG), Darsha Amarthalingam (Public Health Service Amsterdam)

Conflict of interest

None declared.

Authors' contributions

Kimberley Benschop performed the analyses of the data and wrote the paper. Felix Geeraedts, Barbara Beuvink, and Silke Spit performed the analysis of the data and are conducting further clinical and epidemiological investigations on the provincial cluster. Ewout Fanoy was responsible for the communication of the increase. Kimberley Benschop, Eric. Claas, Suzan Pas, Rob Schuurman, Jaco Verweij, Sylvia Bruisten, Katja Wolthers, and Hubert Niesters were responsible for collecting and recording the data in VIRO-TypeNed. All other authors reviewed the paper critically, and comments and suggestions were incorporated in the final version by Kimberley Benschop.

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A norovirus intervariant GII.4 recombinant in Victoria, Australia, June 2016: the next epidemic variant?

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Citation style for this article:
Bruggink L, Catton M, Marshall J. A norovirus intervariant GII.4 recombinant in Victoria, Australia, June 2016: the next epidemic variant?. Euro Surveill. 2016;21(39):pii=30353. DOI: http://dx.doi.org/10.2807/1560-7917.ES.2016.21.39.30353

Article submitted on 05 September 2016 / accepted on 29 September 2016 / published on 29 September 2016

A norovirus recombinant GII.P4_NewOrleans_2009/GII.4_Sydney_2012 was first detected in Victoria, Australia, in August 2015 at low frequency, and then re-emerged in June 2016, having undergone genetic changes. Analysis of 14 years' surveillance data from Victoria suggests a typical delay of two to seven months between first detection of a new variant and occurrence of a subsequent epidemic linked to that variant. We consider that the current recombinant strain has the potential to become a pandemic variant.

This study reports the emergence of a GII.4 intervariant recombinant of GII.P4_NewOrleans_2009 (ORF1) with GII.4_Sydney_2012 (ORF2). This new recombinant – first detected in Victoria, Australia, in August 2015, then re-emerged, with genetic changes, in June 2016 – has been the causative agent in the majority of norovirus gastroenteritis outbreaks in Victoria since its remergence. It is proposed that the pattern of emergence of this strain renders it a potential candidate to become the next GII.4 pandemic variant.

Norovirus strains can be highly adaptable, escaping herd immunity and thus continuing to infect the community over long periods. There is evidence that the emergence of new global epidemic variants and their subsequent global spread is rapid, with almost simultaneous detection worldwide [1-4]. GII.4 noroviruses cause ca 70–80% of all human norovirus-associated gastroenteritis worldwide [5] and pandemics of norovirus infection occurred in 1996, 2002, 2004, 2006, 2009 and 2012, all caused by the emergence of new GII.4 variants [1,6]. No new pandemic strain has emerged since the GII.4_Sydney_2012 variant [5], which has been the predominant strain in Victoria, Australia, since its emergence in 2012, although there is a report [7] of an altered form of the Sydney_2012 variant (referred to as the Sydney_2015 variant) detected over the past 12 months in the United States. The report [7] appears to be based only on partial capsid sequence and it is unclear whether Sydney_2015 is the GII.P4_NewOrleans_2009/GII.4_Sydney_2012 recombinant.

Origin of faecal material for norovirus testing

The Victorian Infectious Diseases Reference Laboratory (VIDRL) is the main public health laboratory for viral identification in the State of Victoria, Australia. Faecal specimens collected from gastroenteritis outbreaks are routinely sent to VIDRL for norovirus testing [8]. An outbreak of gastroenteritis was defined as an incident, apparently associated with an event or location, in which four or more individuals had symptoms of gastroenteritis. During 14 years (2002–15), VIDRL has received a mean of 1,296 faecal specimens per year from gastroenteritis outbreaks. These specimens are from a mean of 273 outbreaks per year, with norovirus detected in a mean of 177 outbreaks per year.

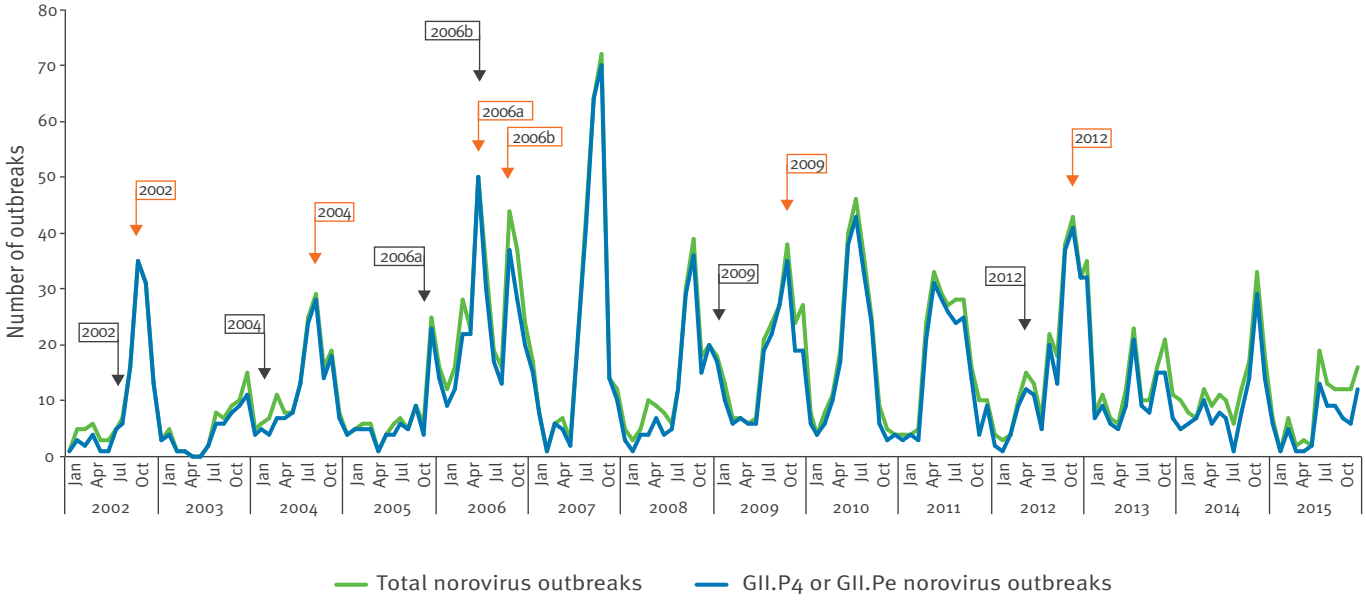
Detection of norovirus and sequencing protocols

Faecal specimens were processed as described previously [8] and then tested by an ORF1 reverse transcription (RT)-PCR that detects both GI and GII norovirus [8]. Additionally, a GII ORF2 RT-PCR was also performed on one specimen from each gastroenteritis outbreak [8]. Where ORF1 and ORF2 testing provided different genotypes, an ORF1-ORF2 bridging PCR was performed to try and confirm recombination status [9]. Full capsid sequence was obtained using primers developed by Kim et al. [10].

Nucleotide sequencing and phylogenetic analysis were carried out as described previously [11] using the software MacVector v15.0, Phylip v3.695 and FigTree v1.4.2. Genotype analysis also made use of the norovirus genotyping tool [12,13]. Information presented on norovirus gastroenteritis outbreak periodicity during 2002 to 2015 (Figure 1) and the associated GII.4 variant information (Figure 1, Table) made use of sequencing

Figure 1

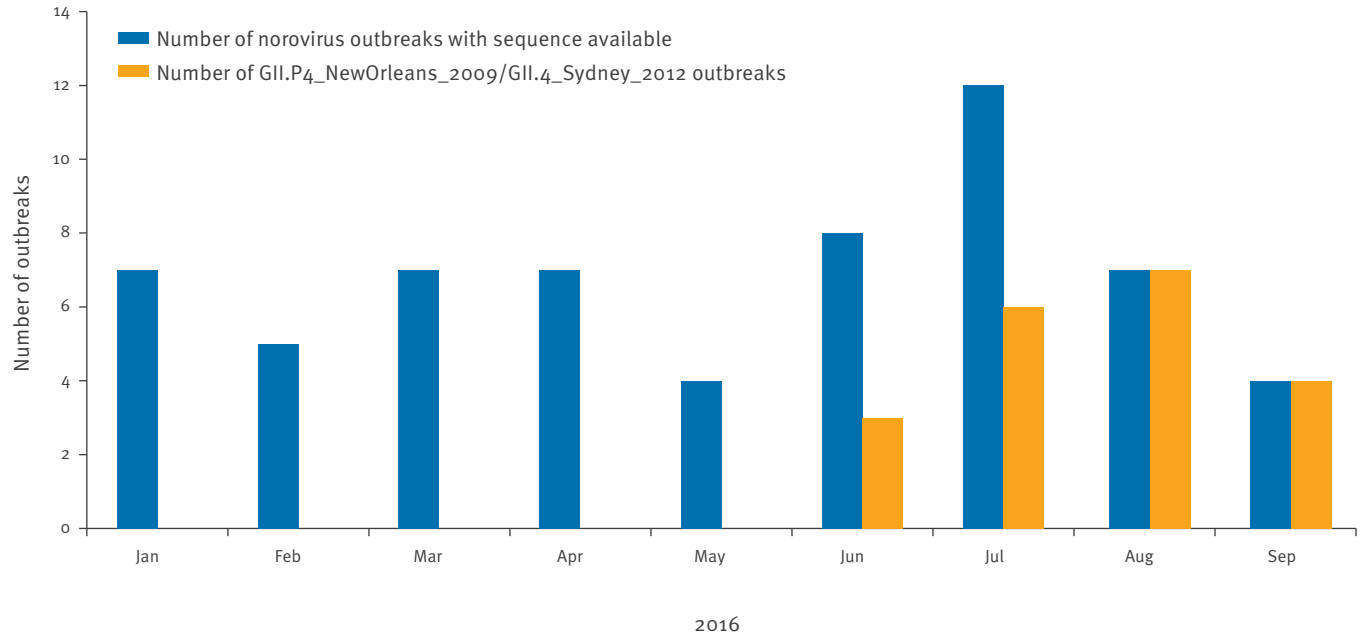
Norovirus gastroenteritis outbreak periodicity, Victoria, Australia, 2002–15 (n = 2,473)



Monthly number of norovirus gastroenteritis outbreaks in the State of Victoria, Australia, from 2002 to 2015 (based on ORF1 reverse transcription (RT)-PCR data). The total number of norovirus outbreaks is shown, as well as the outbreaks where GII.P4 or GII.Pe (ORF1) was detected. It is notable that in most years an epidemic peak occurred in the latter half of the year (spring–summer in Australia). An epidemic 'peak' was considered to be three consecutive months of the highest number of norovirus gastroenteritis outbreaks in a calendar year, except in 2006 where there were two epidemic peaks of similar size [14]. Black arrows indicate the first detection of a new epidemic GII.4 variant (Farmington_Hills_2002, Hunter_2004, Yerseke_2006a, Den Haag_2006b, NewOrleans_2009 and Sydney_2012). Orange arrows indicate the first epidemic peak caused by a new epidemic GII.4 variant. This graph makes use of sequencing information from previous studies in our laboratory [11,14] as well as inclusion of novel data.

Figure 2

Norovirus gastroenteritis outbreaks in Victoria, Australia, 2016[a] (n = 61)

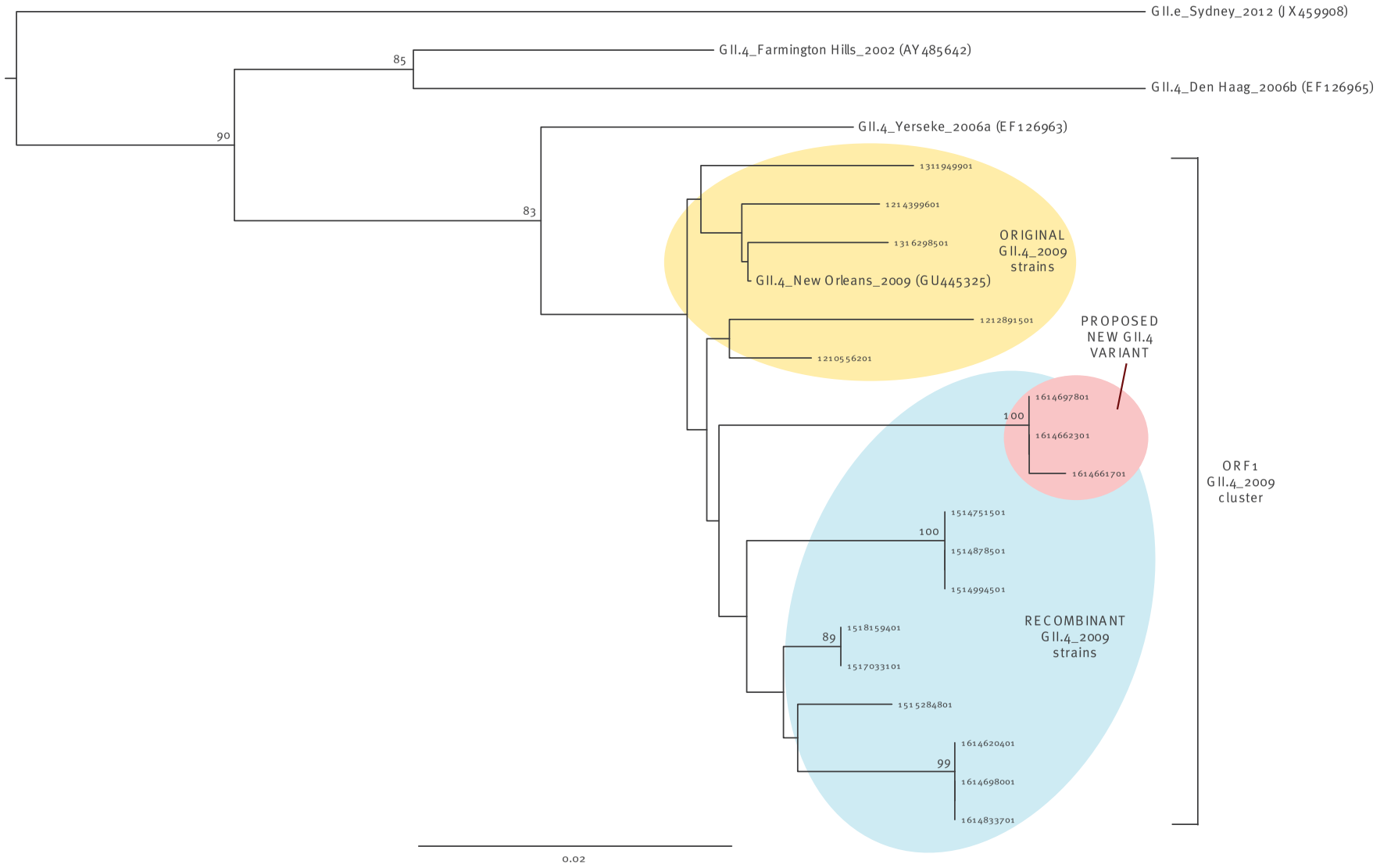


Monthly number of norovirus gastroenteritis outbreaks in the state of Victoria, Australia, 2016 (based on ORF1 reverse transcription (RT)-PCR and sequencing data). The total number of norovirus outbreaks with sequence data available is shown, as well as the number of outbreaks where the GII.P4_NewOrleans_2009/GII.4_Sydney_2012 recombinant was detected.

[a] Data up to 20 September 2016.

Figure 3

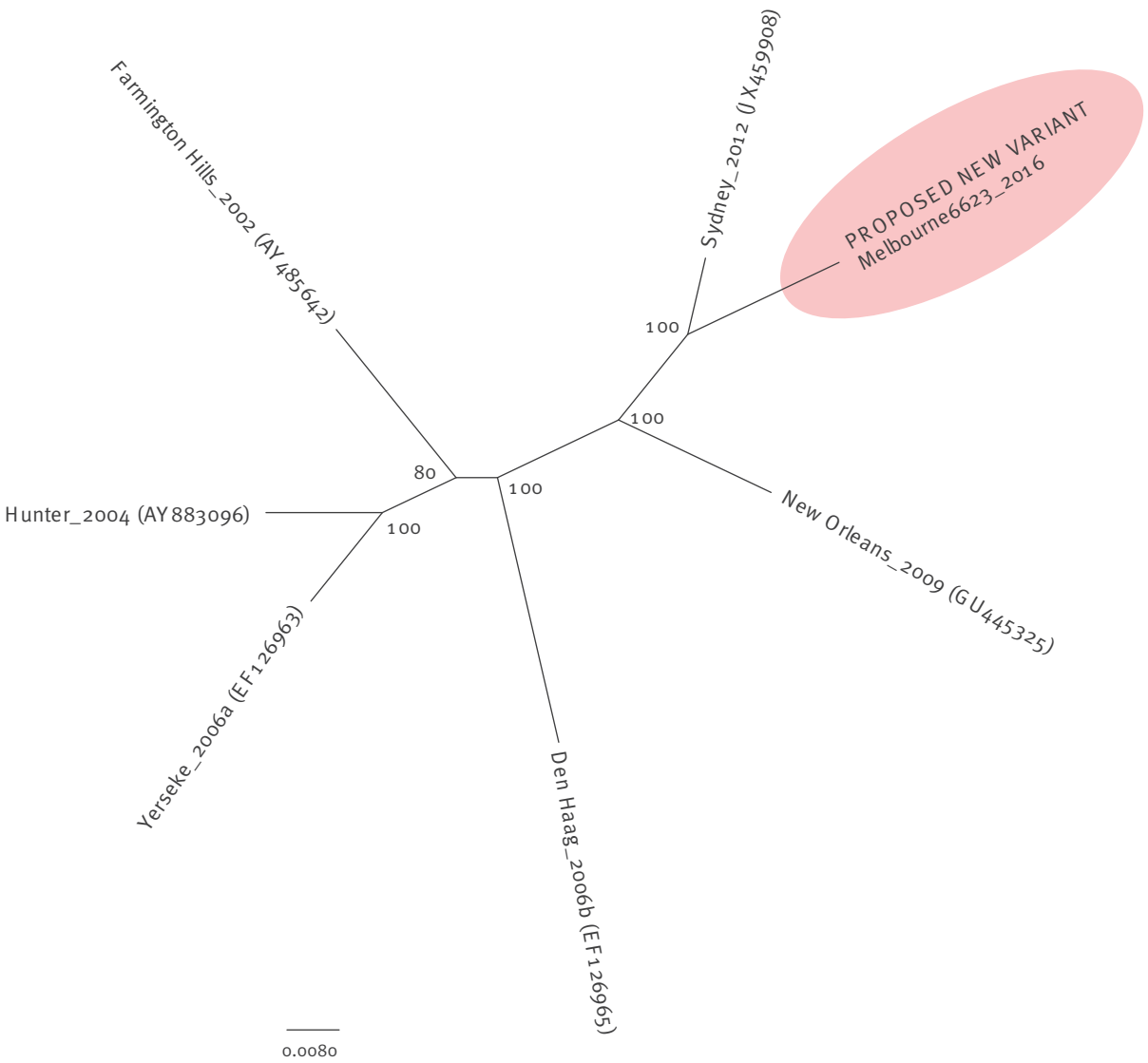
Phylogenetic tree of an ORF1 fragment of norovirus GII.4 strains



DNA (dist) Kimura phylogenetic tree of a 440 bp norovirus ORF1 fragment of norovirus GII.4 strains corresponding to positions 4,484–4,923 of reference strain GII.4_Sydney_2012 (JX459908). Yellow highlights the branch containing the original strain GII.4_NewOrleans_2009 (GU445325) as well as representative specimens of that genotype from 2012 and 2013. Blue highlights the branch containing the recombinant GII.P4_NewOrleans_2009/GII.4_Sydney_2012 with specimens from 2015 and 2016. Red highlights the branch containing the proposed new variant that has altered slightly and is branching away from the recombinant form. The figures on the branches represent bootstrap values (%) after resampling 1,000 datasets. Only bootstrap values≥70% are shown. The scale marker represents substitutions per site.

Figure 4

Phylogenetic tree of the full capsid sequence of norovirus GII.4 variant reference strains



DNA (dist) Kimura phylogenetic tree of GII.4 norovirus full capsid sequence of norovirus GII.4 variant reference strains corresponding to positions 5,085–6,707 (1,623bp) of reference strain GII.4_Sydney_2012 (JX459908). Red highlights the branch containing the potential new variant that has altered slightly and is branching away from the parent strain GII.4_Sydney_2012 (JX459908). The figures on the branches represent bootstrap values (%) after resampling 1,000 datasets. Only bootstrap values ≥ 70% are shown. The scale marker represents substitutions per site.

information from previous studies in our laboratory [11,14] as well as inclusion of novel data.

Norovirus periodicity and GII.4 variants

Although norovirus is detected throughout the year, there is generally one norovirus epidemic peak of outbreaks per calendar year. An epidemic 'peak' was considered to be three consecutive months of the highest number of norovirus gastroenteritis outbreaks in a calendar year, except in 2006 where there were two epidemic peaks of similar size [14]. During 2002 to 2015 there was a single major yearly epidemic peak, except in 2006 when there were two (Figure 1). Norovirus epidemics are generally linked to a GII.4 genotypic variant, with a new variant typically emerging every two to three years at a time point between annual peaks of norovirus detection. In 2006, there was an almost simultaneous emergence of two new epidemic GII.4 norovirus variants, Yerseke_2006a and Den Haag_2006b, with the Den Haag_2006b variant emerging during the Yerseke_2006a epidemic peak.

The emergence of a new norovirus epidemic variant results in an epidemic peak in the number of outbreaks that are predominantly due to the new variant, usually in the year that it emerges and then in a number of

Table

Norovirus GII.4 variants that emerged and led to gastroenteritis epidemics in Victoria, Australia, 2002–15[a]

Norovirus GII.4 variant	Month and year of first detection	First epidemic peak[b]	Delay[c] in months
Farmington_Hills_2002	July 2002	September–November 2002	2
Hunter_2004	February 2004	August–October 2004	6
Yerseke_2006a	December 2005	May–July 2006	5
Den Haag_2006b	June 2006	October–December 2006	4
NewOrleans_2009	January 2009	August–October 2009	7
Sydney_2012	May 2012	October–December 2012	5

[a] This table makes use of sequencing information from previous studies in our laboratory [11,14] as well as inclusion of novel data.

[b] An epidemic 'peak' was considered to be three consecutive months of the highest number of norovirus gastroenteritis outbreaks in a calendar year, except in 2006 where there were two epidemic peaks of similar size [14].

[c] Time delay from first detection to the beginning of the first epidemic peak.

subsequent years, until a successor variant emerges. The Yerseke_2006a variant only caused the first epidemic peak in 2006 and was immediately replaced as the predominant variant by the Den Haag_2006b variant. Analysis of the data from all six variants that emerged during 2002 to 2015 shows that there was a delay of between two and seven months from the first detection of a new variant to the time of the first epidemic peak linked to that variant (Figure 1, Table).

A recently emerged new variant is the GII.4 intervariant recombinant of GII.P4_NewOrleans_2009 (ORF1) with GII.4_Sydney_2012 (ORF2). This recombinant was first detected in Victoria in August 2015 and was only detected at low levels in late 2015 (three of 64 outbreaks in August to December 2015). This strain then remained undetected for five months (January to May 2016) and re-emerged in mid-June 2016 (Figure 2). Since its re-emergence in June 2016, this recombinant strain has caused the majority of the norovirus gastroenteritis outbreaks detected in Victoria (6/12 outbreaks in July, 7/7 outbreaks in August and 4/4 outbreaks in September (up to 20 September)). An ORF1-ORF2 sequence of the first detected recombinant strain from 2015 has been lodged in GenBank under accession number KX064756.

Phylogenetic analysis

The recombinant strain differs from the original parent strains in both ORF1 (Figure 3) and ORF2 (Figure 4). Furthermore, the recombinant strain has undergone some minor alterations, making some of the 2016 strains cluster separately on a phylogenetic tree from earlier 2015 and 2016 recombinant strains (Figure 3).
A phylogenetic tree of the full capsid sequence (Figure 4) shows that the new strain differs from all previous major epidemic variants. We consider that this altered recombinant strain holds the potential to be a new epidemic variant.

Examination of the percentage nucleotide similarity of full capsid sequence from past epidemic variants (2002 AY485642, 2004 AY883096, 2006a EF126963, 2006b EF126965, 2009 GU445325, 2012 JX459908) [6] demonstrates that the variants share 91.4–96.5% similarity. The full capsid sequence of one of the first altered recombinant strains from this study (GII.4/Melbourne6623/2016/AUS) detected in June 2016 has been lodged in GenBank (KX767083). The nucleotide similarity of this strain compared with its closest counterpart, Sydney_2012 (JX459908) is 96.3%, which is consistent with the range given above.

GII.4 hypervariable epitopes

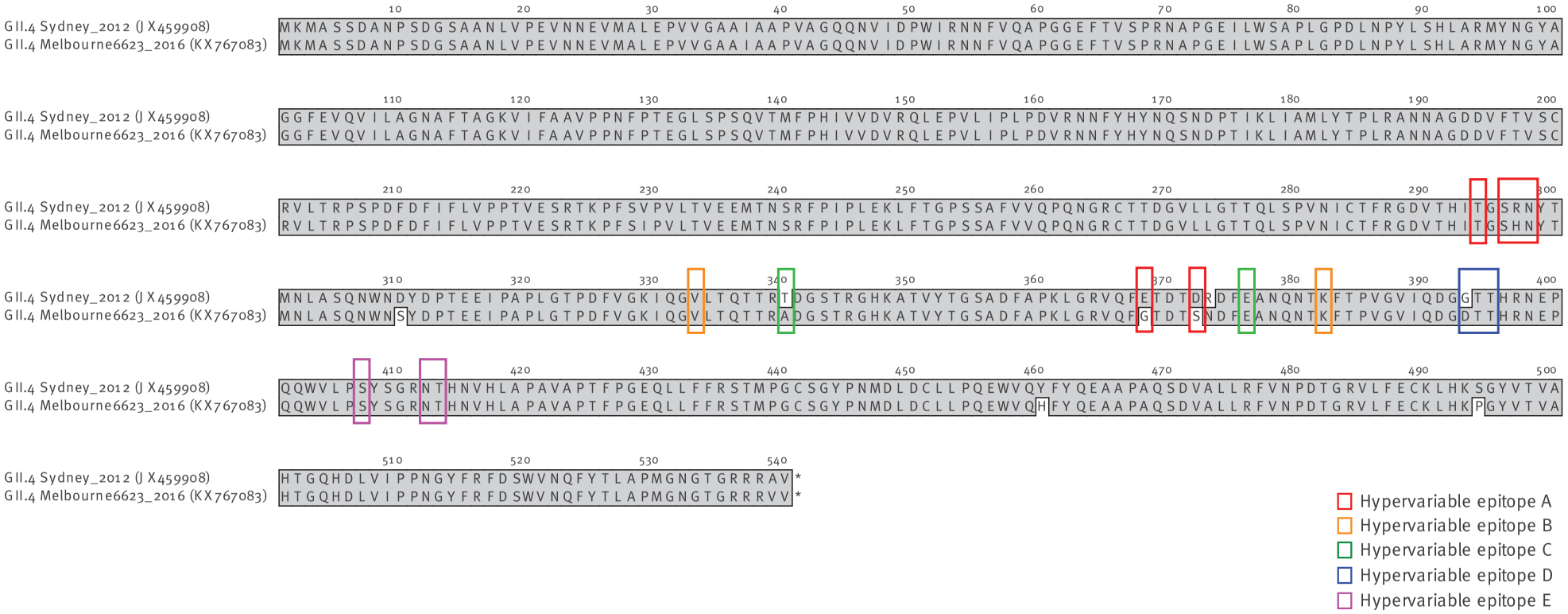
A fundamental study by Lindesmith et al. [15] proposed five hypervariable epitopes (A to E) for GII.4 noroviruses that appear to evolve over time and drive antigenic change, allowing the emergence of new strains that can evade the population's immune response. Amino acid alignment of the full capsid protein of the proposed new variant with that of its closest counterpart, Sydney_2012 (JX459908), shows that of the five epitopes proposed by Lindesmith et al. [15], three have undergone change in the proposed new variant (Figure 5). The three altered epitopes are A, C and D.

Discussion

The classification of new GII.4 variants is based both on phylogenetic analysis and on the presence of the variant becoming epidemic in at least two distinct geographical locations [6]. The recombination of two past epidemic variants (GII.P4_NewOrleans_2009 and GII.4_Sydney_2012), followed by additional evolutionary changes in both ORF1 and ORF2, provides the new strain with genetic novelty. In particular, changes in three of the five hypervariable epitopes proposed by Lindesmith et al. [15] illustrate that the strain has changed at sites critical for the evolution of the virus. The strain has already become predominant between norovirus seasons in outbreaks in Victoria, Australia, over the past three months (July to September). On this basis, we propose it as a candidate new epidemic strain. If past trends are followed, then it would have the potential to also predominate in other parts of the world.

Figure 5

Amino acid alignment of the full capsid protein of the GII.4_Sydney_2012 norovirus reference strain and the proposed new variant detected in June 2016 in Victoria, Australia



Amino acid alignment of norovirus full capsid protein of GII.4_Sydney_2012 (JX459908) and the proposed new variant GII.4_Melbourne6623_2016 (KX767083) from this study. Hypervariable epitopes A to E [15] are outlined.

When 14 years of norovirus gastroenteritis outbreak incidence data are analysed for epidemic GII.4 variants, it can be seen that between two and seven months typically pass between first detection of the variant and the subsequent epidemic. If this new recombinant has undergone enough change to escape herd immunity and become the next epidemic variant, then from its first detection in Victoria in mid-June 2016, the expected epidemic could be any time between mid-August 2016 and January 2017. As the emergence of new global epidemic variants and their subsequent spread is rapid, and they are detected almost simultaneously worldwide [1-4], the rest of the world could also undergo a norovirus epidemic within a similar time frame.

A key observation in this report is the delay between the first appearance of an epidemic norovirus strain and the subsequent epidemic involving that strain. This observation accords well with the recent findings of Allen et al. [16], who suggest that pandemic strains may circulate at low levels in the community up to several years before their global spread. The concept of delay in norovirus epidemic variant circulation is still poorly understood and clearly requires further investigation.

Acknowledgements

We would like to acknowledge the Victorian Department of Health and Human Services for assistance and support in preparation of this manuscript.

Conflict of interest

None declared.

Authors' contributions

Ms Leesa Bruggink performed norovirus testing, sequencing, data analysis and manuscript preparation. Dr Michael Catton reviewed the data and assisted in manuscript preparation. Dr John Marshall performed some laboratory work, reviewed the data and assisted in manuscript preparation.

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French *Aedes albopictus* are able to transmit yellow fever virus

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Citation style for this article:
Amraoui F, Vazeille M, Failloux AB. French Aedes albopictus are able to transmit yellow fever virus. Euro Surveill. 2016;21(39):pii=30361. DOI: http://dx.doi.org/10.2807/1560-7917.ES.2016.21.39.30361

Article submitted on 14 September 2016 / accepted on 29 September 2016 / published on 29 September 2016

We assessed the ability of a French population of *Aedes albopictus* to transmit yellow fever virus (YFV). Batches of 30 to 40 female mosquitoes were analysed at 7, 14 and 21 days post-exposure (dpe). Bodies, heads and saliva were screened for YFV. Infectious viral particles were detected in bodies and heads at 7, 14 and 21 dpe whereas the virus was found in saliva only from 14 dpe. Our results showed that *Ae. albopictus* can potentially transmit YFV.

We assessed the vector competence of *Aedes albopictus* collected in France for a West African strain of yellow fever virus (YFV). Our results show that this temperate population of *Ae. albopictus* was able to deliver virus through saliva 14 days after receiving an infectious blood-meal.

Experimental infection of mosquitoes

A YFV S79-P4 strain isolated in 1979 from a human case in Senegal [1] was passaged twice on newborn mice and two times on C6/36 *Ae. albopictus* cells. Viral stocks were produced on C6/36 *Ae. albopictus* cells.

Ae. albopictus mosquitoes used for the study originated from Bar-sur-Loup, a commune in the department of Alpes-Maritimes, which is in the region of Provence-Alpes-Côte d'Azur in south-east France. Eggs were collected from the field in ovitraps and reared in an insectary for 11 generations (the generation time is approximately 10 days) before experimental infections. Several batches of 200 larvae were reared in pans containing 1 L of dechlorinated tap water and a yeast tablet renewed every two days. Adults were maintained at 28 °C ± 1 °C in 80% relative humidity with a light:dark cycle of 16h:8h. The mosquitoes were fed ad libitum with a 10% sucrose solution. Females were blood fed three times a week on anaesthetised mice (OF1 mice, Charles River laboratories, France). Adult females were exposed to an infectious blood-meal containing 106.2 foci fluorescent units (FFU)/mL of YFV S79-P4 strain mixed with rabbit blood and maintained at 28 °C for 21 days without any additional blood meals.

A total of 30 to 40 exposed mosquitoes were analysed at 7, 14 and 21 days post-exposure (dpe) to estimate the four indices describing the vector competence: (i) the infection rate (IR), which corresponds to the proportion of successfully infected mosquitoes (viral particles detected in bodies) after exposure to an infectious blood-meal among analysed mosquitoes, (ii) the disseminated infection rate (DIR), which measures the proportion of mosquitoes with evidence that the virus crossed the midgut barrier to reach the haemocoel and infected internal organs (infection detected in heads) among infected mosquitoes, (iii) the transmission rate (TR), which estimates the proportion of mosquitoes with the virus present in saliva among mosquitoes able to disseminate the virus in the mosquito haemocoel (examined when calculating DIR), and (iv) the transmission efficiency (TE), which corresponds to the overall proportion of females with the virus present in saliva among the total number of tested mosquitoes. Saliva was collected using the forced salivation technique previously described [2]. Briefly, wings and legs of each mosquito were removed from each mosquito and the proboscis was inserted into a 20 µL tip containing 5 µL of fetal bovine serum (FBS). After 30 to 45 min of salivation, FBS containing saliva was expelled in 45 µL of Dulbecco's modified Eagle medium (DMEM) for further titration. Heads/bodies homogenates and saliva from respective mosquitoes were titrated by focus fluorescent assay on C6/36 *Ae. albopictus* cells as prior described [3].

Vector competence analysis

When analysing the ability of *Ae. albopictus* to be infected at 7, 14 and 21 dpe, IRs remained below 15/40 and were similar regardless of the dpe examined (7dpe: 6/40, 14 dpe: 15/40 and 21 dpe: 8/30; Fisher's exact test: $p = 0.074$). When testing the ability of mosquitoes

to undergo dissemination of the virus beyond the midgut barrier, DIR did not exceed 6/8 as observed at 21 dpe and remained comparable for the three dates post-exposure (7 dpe: 2/6, 14 dpe: 9/15 and 21 dpe: 6/8; Fisher's exact test: p = 0.29).

When examining mosquito saliva for YFV among mosquitoes with a viral dissemination to calculate the TR, we found that the virus could be detected in saliva at 14 dpe (TR=2/9) and 21 dpe (TR=1/6). No virus was detected at 7 dpe. The corresponding TEs for *Ae. albopictus*, which take into account the total number of tested mosquitoes, were two individuals among 40 tested at 14 dpe and one among 30 at 21 dpe. When considering only mosquitoes with infectious saliva (n=3), a mean of 52 viral particles (standard deviation ± 28; n=2 individual mosquitoes' saliva examined) was estimated at 14 dpe and 10 viral particles (1 mosquito's saliva) at 21 dpe. Hence *Ae. albopictus* from southern France was able to transmit a West African YFV from 14 dpe.

In a separate unpublished study (data not shown) that we conducted on *Ae. aegypti*, we found that at 14 dpe, *Ae. aegypti* had an IR of 5/17, a DIR of 2/5 and a TE of 2/17. This may suggest that *Ae. albopictus* mosquitoes might have higher rates of infection and dissemination of the virus in the body (15/40 and 9/15 respectively) than *Ae. aegypti*, albeit a lower TE (2/40).

Background

Yellow fever (YF) is a potentially deadly disease with symptoms including jaundice, enlargement of the liver, and haemorrhage [4]. It is caused by YFV (*Flavivirus, Flaviviridae)*, a virus that was first isolated in West Africa in 1927 [5]. Globally, the heaviest burden of YF is in Africa where the endemic area covers 34 countries and concerns ca 500 million people [6].

Besides genetic differences between seven YFV genotypes identified to date [7], the competence of potential mosquito vectors to transmit the virus may affect the distribution pattern of YF outbreaks. In sub-Saharan Africa, where more than 90% of YF cases occur, three different transmission cycles have been described [4]. In the jungle cycle, YFV can spread between non-human primates by canopy-dwelling mosquitoes such as *Ae. africanus*. The intermediate or savannah cycle involves other mosquito species including *Ae. luteocephalus, Ae. furcifer, Ae. metallicus, Ae. opok, Ae. taylori, Ae. vittatus* and members of the *simpsoni* complex. In areas where this cycle occurs, termed 'zones of emergence', YFV is transmitted from non-human primates to humans. Lastly, the urban cycle involves transmission of YFV between humans by the anthropophilic mosquito *Ae. aegypti*. In South America, YFV circulates exclusively in a jungle cycle involving *Haemagogus janthinomys* and *Sabethes chloropterus* mosquitoes and non-human primates [4]. The virus is absent in Asia although local *Ae. aegypti* are susceptible to the virus [8].

Since 1937, YF can be prevented through immunisation provided by the 17D vaccine; one dose confers a protective immunity for life and more than 650 million doses have been distributed in the past 75 years [9]. In endemic areas for YF however, funds are lacking to stimulate YFV vaccine production and accelerate vaccination campaigns, and human cases continue to be recorded annually. Moreover, during the past 20 years, at least one annual YF outbreak has been reported in Africa, mainly in West Africa (East and Central African countries are usually less affected). In such outbreaks, human cases are mainly associated with mass migrations of non-immunised people who have been exposed to YF in endemic areas, reminding that YF is still a major public health problem.

On 21 January 2016, an outbreak of YF occurred Angola [10]. With more than 3,000 suspected cases and 300 deaths as of 10 June 2016, the country is facing the most important urban YF outbreak observed so far in Africa [11]. Despite a slow decrease in the number of cases in Angola since the end of March 2016 [12], YFV circulation meanwhile continued to expand to neighbouring countries, such as Congo [13] and Uganda [14]. In Congo, 700 suspected cases with 63 deaths were recorded on 31 May 2016 while in Uganda, 30 cases including seven deaths were reported from 26 March to 18 April 2016. Most cases were found in cities suggesting that transmission implicates urban vectors, mainly *Ae. aegypti*. Imported YF cases from Angola were also later confirmed in Kenya [15] and China [16,17], highlighting that while the YF vaccine is very effective, there is a potential risk for unvaccinated travellers from endemic areas to further export the virus.

Discussion

The establishment of a local YF transmission cycle outside endemic areas is related to competent *Aedes* mosquitoes, active all year long in tropical regions and during the warm period in temperate areas. The mosquito species *Ae. albopictus* is present in 20 European countries [18], and a strain of this species (Houston) in the United States has been previously reported to be a competent vector for YFV [19]. Hence travellers returning to Europe from countries where a YF outbreak is occurring could be a source of infection for local strains of *Ae. albopictus*. We therefore assessed the competence of *Ae. albopictus* mosquitoes from the south of France for a West African strain of YFV.

The virus was detected at 14 dpe in saliva of the French *Ae. albopictus* mosquitoes at a rate of two mosquitoes in 40, a relatively low TE. While this is reassuring, a low vector competence can on the other hand contribute to select for virulent virus strains capable of eliciting high viraemia in humans [20] and causing more severe clinical symptoms [21]. Moreover although our results point to a low TR (2/9) for YFV, the anthropophilic nature of *Ae. albopictus* mosquitoes and their high densities in urban areas may allow them to be a vector of YFV.

Concerning the virus strain assessed in this study, the West African YFV strain should not be very genetically distant from the other six genotypes with ca 9% amino-acid divergence between strains, indicating genetic stability of YFV genotypes [7]. However, small genetic changes in the viral genome may change the vector competence.

As Europe has faced YF outbreaks in the past [22], the last being recorded in Gibraltar in 1905, a risk of importation of YF into Europe is to be considered. Although so far there have been hardly any reports from Europe of imported YF cases, many imported cases of chikungunya and dengue, two other arboviral diseases, have been documented [23]. If YF follows the same path as dengue and chikungunya, which have a greater number of imported cases, a local transmission of YF in temperate regions where *Ae. albopictus* is established becomes a plausible scenario, underlining the need for continued vigilance for YF.

Acknowledgements

We would like to thank Pascal Delaunay for the mosquito strain.

Conflict of interest

None declared.

Authors' contributions

FA designed and performed the research. MV produced viral stocks. ABF designed the research, analysed the data and wrote the paper.

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VIRO-TypeNed, systematic molecular surveillance of enteroviruses in the Netherlands between 2010 and 2014

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Citation style for this article:
Benschop KSM, Rahamat-Langendoen JC, van der Avoort HGAM, Claas ECJ, Pas SD, Schuurman R, Verweij JJ, Wolthers KC, Niesters HGM, Koopmans MPG, on behalf of VIRO-TypeNed. VIRO-TypeNed, systematic molecular surveillance of enteroviruses in the Netherlands between 2010 and 2014. Euro Surveill. 2016;21(39):pii=30352. DOI: http://dx.doi.org/10.2807/1560-7917.ES.2016.21.39.3035

Article submitted on 22 October 2015 / accepted on 23 May 2016 / published on 29 September 2016

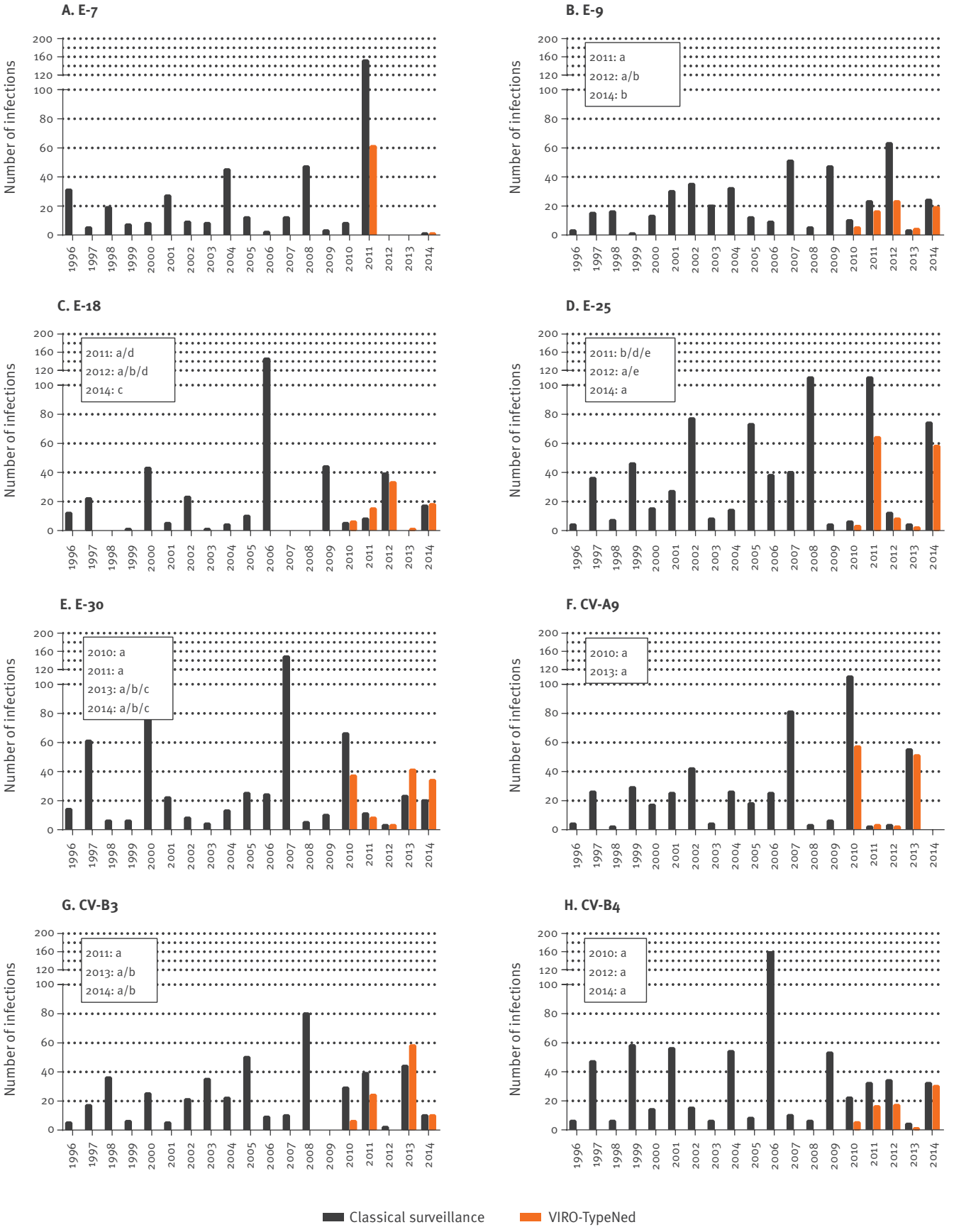
VIRO-TypeNed is a collaborative molecular surveillance platform facilitated through a web-based database. Genetic data in combination with epidemiological, clinical and patient data are shared between clinical and public health laboratories, as part of the surveillance underpinning poliovirus eradication. We analysed the combination of data submitted from 2010 to 2014 to understand circulation patterns of non-polio enteroviruses (NPEV) of public health relevance. Two epidemiological patterns were observed based on VIRO-TypeNed data and classical surveillance data dating back to 1996: (i) endemic cyclic, characterised by predictable upsurges/outbreaks every two to four years, and (ii) epidemic, where rare virus types caused upsurges/outbreaks. Genetic analysis suggests continuous temporal displacement of virus lineages due to the accumulation of (silent) genetic changes. Non-synonymous changes in the antigenic B/C loop suggest antigenic diversification, which may affect population susceptibility. Infections were frequently detected at an age under three months and at an older, parenting age (25–49 years) pointing to a distinct role of immunity in the circulation patterns. Upsurges were detected in the summer and winter which can promote increased transmissibility underlying new (cyclic) upsurges and requires close monitoring. The combination of data provide a better understanding of NPEV circulation required to control and curtail upsurges and outbreaks.

Introduction

Enteroviruses (EVs) are widespread viruses circulating globally. More than 100 types, classified to the four species A to D within the genus *Enterovirus* of the *Picornaviridae* family, are known to infect and cause disease in humans [1] The epidemiology of enteroviruses is characterised by the occurrence of seasonal peaks in the summer and temporal outbreaks that can be associated with life-threatening EV infections [2]. Clinical manifestations vary and range from asymptomatic or mild respiratory or gastrointestinal symptoms to severe and even fatal cases of myocarditis, neonatal sepsis, and central nervous system infections [3,4]. Severe and fatal cases often occur in children younger than five years or immunocompromised individuals [5-11]. Unfortunately, treatment options are limited and specific antivirals are not yet available [12,13]. Enteroviruses evolve by genetic diversification and recombination [14-17], which may affect their virulence [18-30]. In the Asian Pacific Region, EV-A71 (genotype C4) has been causing large outbreaks of hand, foot and mouth disease (HFMD) with severe complications since 2008 [22,23]. In a recent outbreak in the United States (US) and in Europe, an EV-D68 variant has been associated with outbreaks of severe respiratory disease and possibly paralysis [24-30]. While vaccination is a proven control strategy for some picornaviruses (poliovirus (PV), hepatitis A virus), little is known about the impact on population level of non-polio enteroviruses (NPEVs), even though these are among the most common endemic viruses. Knowledge on trends of NPEV illness comes from the decade-old EV surveillance programmes that have been implemented globally, where typing of enteroviruses from clinical samples from patients with polio-like illness is a cornerstone in the PV eradication campaign. Typing has historically been done by use of antigenic characterisation of virus isolates from routine diagnostic laboratories that used

Figure 1

Distribution of endemic enterovirus types, the Netherlands, 1996–2014 (classical surveillance; n = 4,098) and 2010–2014 (VIRO-TypeNed; n = 714)



Lineages are indicated in boxes in the graphs. Black bars indicate classical surveillance. Orange bars indicate VIRO-TypeNed data.

cell culture as the primary diagnostic method. Now laboratories are switching more and more to molecular detection and typing methods [31,32]. A major drawback of the widespread introduction of fast molecular diagnostic methods is that the surveillance spin-off of NPEV from the EV surveillance programme is no longer routinely available, and that molecular typing is dedicated to a few larger diagnostic and university hospital centres. To compensate for this, we have launched a collaborative molecular surveillance programme in 2010, in which sequence-based surveillance was introduced (VIRO-TypeNed) [33].

Here, we present an analysis of data submitted through this novel surveillance system from 2010 through 2014, which provides a better understanding of NPEV circulation in relation to seasonal epidemics and outbreaks.

Methods

Sampling and laboratory enterovirus diagnostic testing

Stool, respiratory, cerebrospinal fluid (CSF), blood and vesicle fluid samples from patients of different age groups, admitted to or visiting the hospital with an EV-associated illness, were sent to the clinical virology laboratories for testing. Symptoms varied from mild to severe respiratory illness, fever and appearance of vesicles to central nervous symptoms such as meningitis. Enterovirus testing were done by enterovirus-specific PCR tests [34,35], which are based on the conserved 5'UTR and enable detection of both PV and NPEVs, including those that do not grow in cell culture [36,37].

Virus characterisation of 5'UTR-positive samples

Positive samples were characterised directly from clinical material or culture-positive samples by sequencing the VP1 gene [31,38-40]. The VP1 partial sequences obtained were used as input in the typing tool with an automated algorithm to assign the species and (sub) type of the sequences entered [41]. In addition, samples that could not be typed, in particular those with suspicion of PV infection, were sent to the reference laboratory (National Institute for Public Health and the Environment (RIVM)) for cultivation on the PV-specific cell line L20B to document the absence of wild-type PV circulation. Laboratories that do not perform typing of EVs are encouraged to send EV isolates or 5'UTR-positive samples to the RIVM for exclusion of PV and further characterisation.

Reporting of data for EV surveillance by VIRO-TypeNed

The concept of VIRO-TypeNed has been described in detail elsewhere [33]. In short, participating Dutch clinical virology laboratories and the RIVM agreed on a consensus typing method described by Nix et al. [42] and on sharing of anonymised data in compliance with privacy rules via a secured web-based database. For each patient with a positive 5'UTR sample, at least one sequence of the VP1 gene generated by the Nix method is shared [40,42]. Sequences generated by other protocols can be included as well [43], but can only be included in the phylogenetic analysis when the region is compatible with the region generated with the Nix protocol. The VIRO-TypeNed platform includes a sequence-based typing tool with an automated algorithm to assign the species and (sub)type of the sequences entered, thus assuring comparability between the laboratories [41]. When available, a minimum set of clinical and epidemiological data are included with the submitted sequences, consisting of age, sex, date and type of sample, hospitalisation, travel history, clinical symptoms (skin, neurological, respiratory, enteric and other) and mortality.

Data analysis

We analysed data submitted during the first five years of the VIRO-TypeNed project for trends, clusters and genetic diversity of common enteroviruses. Using data from the classical enterovirus surveillance containing data dating back to 1996 [32], the circulation patterns of the types were defined as endemic cyclic or as epidemic: (i) types with an endemic cyclic pattern of circulation were characterised by (predictable) seasonal increases every two to four years, with low detection levels in intervening years and (ii) types with an epidemic pattern were characterised by a unique outbreak in a given year while being rare (detection level $n < 10$) for at least 10 years before the given year [32].

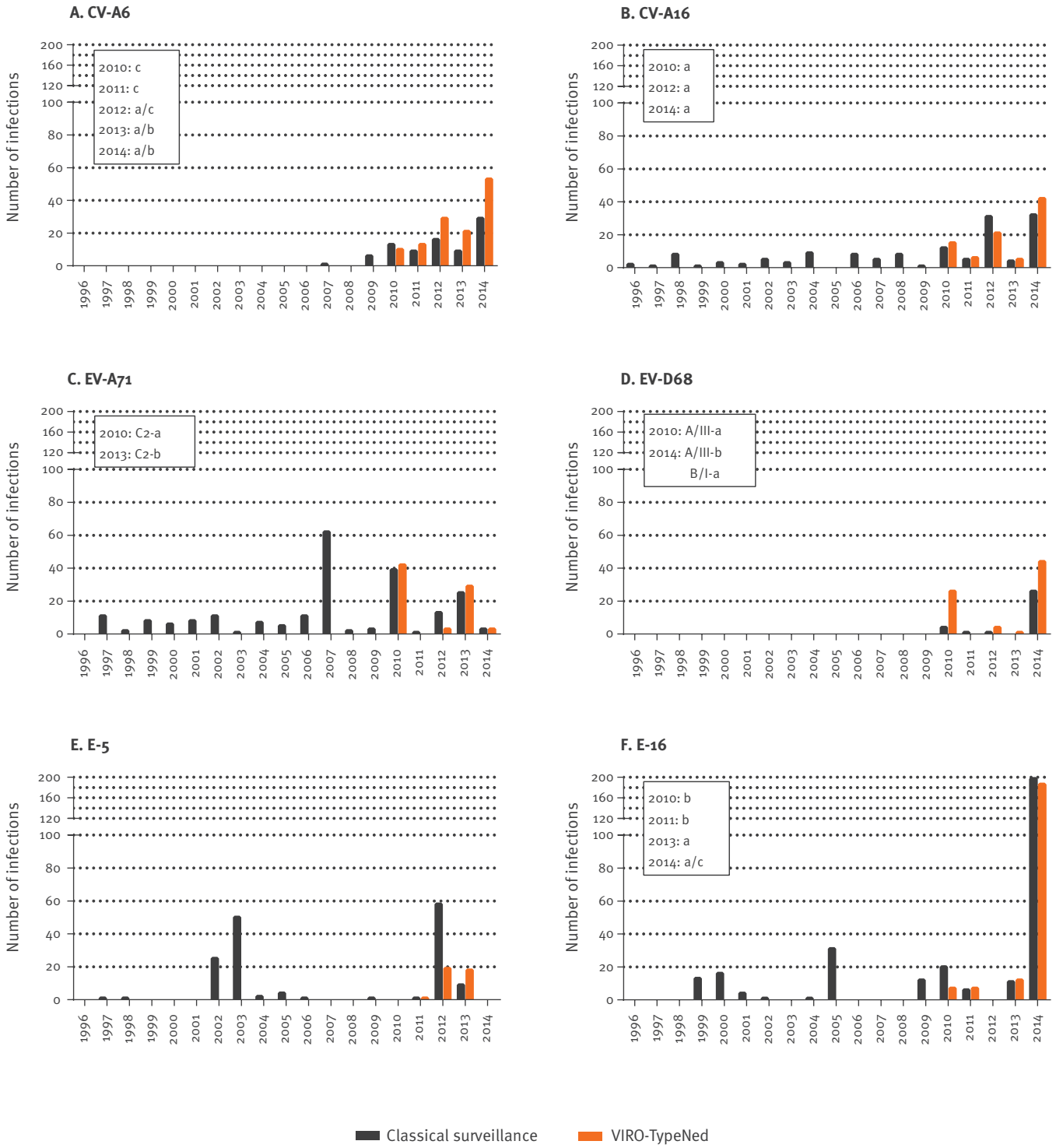
To identify potential viral factors underlying the circulation patterns, all available partial VP1 gene sequences, which included the putative immunogenic B/C loop [44], were aligned and analysed for nucleotide and amino acid changes between lineages using the Simmonics sequence editor [45]. Pairwise distribution based on the nucleotide sequences of VP1 was calculated by MEGA6 [46] and used to set a demarcation cut-off to define lineages [47,48]. With the exception of EV-A71, classification of many NPEVs into lineages is not standardised. In cases where there is no uniform accepted lineage classification, lineages were designated alphabetically. EV-D68 lineages were designated as proposed by Tokarz et al. (A–C) [49] and Meijer et al. (I–III) [50].

Results

In the period from 2010 through 2014, six diagnostic and university hospital laboratories from different parts of the Netherlands participated in the network (University Medical Center Groningen, Leiden University Medical Center, Erasmus Medical Center Rotterdam, University Medical Center Utrecht, St Elisabeth Hospital Tilburg and Academic Medical Center Amsterdam). The RIVM reported genotyping results of samples referred by 17 of 26 virology laboratories across the Netherlands that do not perform typing. A total of 1,917 EV-positive samples that had been genotyped were reported during the study period. The EV-B species was the most frequently detected species in all five years taken

Figure 2

Distribution of epidemic enterovirus types, the Netherlands, 1996–2014 (classical surveillance; n = 938 and 2010–2014 (VIRO-TypeNed; n = 619)



Lineages are indicated in boxes in the graphs. Black bars indicate classical surveillance. Orange bars indicate VIRO-TypeNed data.

together, accounting for 70.3% (n = 1,347) of the viruses detected. The EV-A species comprised the second major species (24.4%, n = 467). The EV-C species and EV-D species (all identified as type EV-D68) accounted for 1.4% (n = 25) and 4% (n = 77), respectively, of the viruses detected. Oral PV vaccine (OPV) strains were detected in four patients; two cases in 2010 with OPV2 and OPV3, respectively, and two cases in 2011 also with OPV2 and OPV3, respectively. The five most commonly reported types and their ranking varied each year and accounted in total for 49–63% of the infections identified (Table).

Endemic circulation

Types that were characterised as endemic cyclic were E-25 (n = 136), E-30 (n = 123), CV-A9 (n = 113), CV-B3 (n = 98), E-18 (n = 73), CV-B4 (n = 69), E-9 (n = 67) and E-7 (n = 62); they occurred with sharp peaks every two to four years (Figure 1). While there was no clear pattern indicative of evolution, phylogenetic clustering (data not shown) suggested the circulation of distinct genetic lineages that were defined by genetic changes in different motifs of the VP1 gene. E-9, E-18, E-25, E-30 and CV-B3 lineages were temporally defined (Figure 1), indicating continuous temporal displacement of variants. CV-A9 and CV-B4 showed no sequence divergence between the years (Figure 1). All E-7 strains were collected in 2011 and were genetically similar. For all types, we found that the genetic changes were primarily silent. In addition to these silent changes, E-25, CV-B3 and E-9 variants showed amino acid substitutions within the B/C loop, suggesting antigenic diversification over time. E-25 strains encoded a valine (V, lineage b and d) or threonine (T, lineages a and e) at position 78 and an aspartic acid (D, lineage b and a) or asparagine (N, lineage d and e) at position 86 of the E-25 VP1 protein (amino acid numbering based on GenBank accession number HM03119). CV-B3 strains encoded a lysine (K, lineage a) or asparagine (N, lineage b) at position 85 of the CV-B3-VP1 protein (numbering based on GenBank accession number JX312064) and E-9 carried an asparagine (N, lineage a) or aspartic acid (D, lineage b) at position 84 of the E-9 VP1 protein (numbering based on GenBank accession number AF524866) [44,51,52].

Epidemic circulation

Types that were characterised as epidemic were E-16 (n = 212), CV-A6 (n = 126), CV-A16 (n = 89), EV-A71 (n = 77, of which 69 were C2), EV-D68 (n = 77) and E-5 (n = 38). E-16 was frequently reported in 2014 and was detected in 29% of the infections in the summer of 2014, which was twice as much than any other type detected in previous years (Figure 2).

Of interest is that CV-A6, CV-A16 and EV-A71 has continuously been detected in an endemic cyclic pattern since 2010 (Figure 2). Sequence divergence between CV-A6 lineages and EV-A71 C2 sublineages were primarily silent and were temporally defined (Figure 2), indicating the same continuous displacement of variants as seen for the endemic types. In contrast, CV-A16 variants did not display any significant sequence variations defining temporal clustering (Figure 2).

The shift from epidemic to endemic cycle was observed also for EV-D68. An upsurge of EV-D68 has been observed in 2010, after very low frequency for 14 years, and again in 2014 (Figure 2). The EV-D68 strains from 2010 and 2014 clustered as temporally defined sublineages within the previously assigned lineages A/III and B/I (Figure 2). Sequence divergence between the two lineages showed amino acid substitutions within the B/C loop; the variants of the 2014 B/I lineage were found to encode D90, T92 and alanine (A)95 of the VP1 protein (amino acid numbering based on GenBank accession AB061487). The variants of the 2010 and 2014 A/III lineages were found to encode N90, A92 (2010, lineage a) or T92 (2014, lineage b), and glutamic acid (E)95 (Figure 2).

Patient characteristics

Using additional patient data submitted to VIRO-TypeNed, we analysed factors such as age of infection, sex and clinical symptoms and their influence on the occurrence of endemic and epidemic patterns of the different types. The female:male sex ratio varied considerably between the types and there was no difference between endemic and epidemic types. Infections were frequently or equally found among girls for the endemic types CV-A9 (ratio: 0.9) and CV-B4 (ratio: 0.9), and the epidemic types EV-D68 (ratio: 0.7) and CV-A6 (ratio: 1). For most types, infections were frequently found among boys for the endemic types E-30 (ratio: 1.1), CV-B3 (ratio: 1.2), E-25 (ratio: 1.3), E-5 (ratio: 1.9), E-18 (ratio: 1.7), E-9 (ratio: 1.5), E-18 (ratio: 1.7) and E-7 (ratio: 2.4), and the epidemic types E-16 (ratio: 1.1), CV-A16 (ratio: 1.1) and EV-A71 (ratio: 1.5).

Overall, infections were detected in cases of all ages (‹ 28 days to 85 years) with a majority of cases younger than five years (n = 1,067/1,249; 56%). Cases infected with the epidemic types E-5 and E-16 were predominantly younger than three months (n = 18/38; 47% and n = 159/212; 75%, respectively). In contrast to the EV-B types characterised as either endemic or epidemic, cases infected with EV-A types were predominantly one to two years of age (25–35%): CV-A6 (n = 38/126), CV-A16 (n = 24/89), EV-A71 (n = 19/77) and the EV-D type EV-D68 (n = 25/77). A number of viruses were also frequently found in adults (at parenting age, 25–45 years) in 3–20% of the cases: E-30 (n=15/123), CV-A9 (n=11/113), E-18 (n = 8/73), CV-A6 (n = 14/126), CV-A16 (n = 5/89) and EV-A71 (n = 3/77). Adult infections with EV-D68 were also frequently found among cases aged 45 to 65 years (n = 12; 16%). No adults with E-25, CV-B3 and E-9 infections were identified. Adults infected with other EV-B comprised less than 1% of the infections. Clinical information was reported for only 5–30% of the cases. Nonetheless, it was found that all HFMD disease reports were attributed to EV-A infections (10/25, p = 0.003). Neurological symptoms were statistically more frequently reported among EV-B-infected cases (85/129) compared with only a few EV-A-infected cases (8/21) (p = 0.027).

Seasonal distribution

Because typing data were linked to date of isolation, VIRO-TypeNed enabled direct analysis of seasonal trends of new upsurges of the common types as well as trends between the types. A clear seasonal distribution was observed in the years 2010, 2013 and 2014, with 15–29% of the EV infections observed in July and August of those years. In some years, there were clear seasonal peaks in winter, contrary to the summer peaks

Table

The five most frequent non-polio enterovirus infections collected by VIRO-TypeNed, the Netherlands, 2010–2014 (n = 1,917)

Ranking	2010	2011	2012	2013	2014
1	CV-A9	E-25	E-18	CV-B3	E-16
2	EV-A71	E-7	CV-A6	CV-A9	E-25
3	E-30	CV-B3	E-9	E-30	CV-A6
4	EV-D68	CV-B4	CV-A16	EV-A71	CV-A16
5	CV-A16	E-9	E-5	CV-A6	EV-D68

The ranking is based on the number of cases recorded.

that are considered typical for enteroviruses. In 2011 and 2012, most EV infections detected in those years were found in the late fall and winter 2011/12 (October to February; detection ranged from 16% in October 2011 to 9% in February 2012). In contrast, not many summer infections were reported in those years (11% in July 2011 and 6% in August 2012). When an upsurge was detected after more than 10 years of low EV activity, the epidemic types CV-A6, CV-A16, EV-D68 and E-5 were predominantly detected in the winter. For CV-A6, CV-A16 and EV-D68, the seasonality shifted towards summer and fall in the following years.

Discussion

In this paper, we describe the systematic surveillance of NPEVs for the Netherlands through the VIRO-TypeNed system, which is based on molecular typing of pathogens.

By analysing virus sequence data in combination with epidemiological and patient data, we show for the first time in a standardised manner the circulation patterns of NPEVs in the Netherlands enabling a better understanding of NPEV circulation, which is required to control and curtail outbreaks and upsurges. With the knowledge on the endemic cyclic patterns, a rise in the number of positive cases two to four years after the last upsurge warrants vigilance because it could indicate an imminent upsurge/outbreak [53]. Of interest is the shift from an epidemic to an endemic pattern for CV-A6, CV-A16, EV-A71 and EV-D68; this should be taken into account when monitoring rare types. For the EV-A viruses, the observed shift could be a surveillance artefact due to the change in detection methods from culture to molecular, as molecular methods have increased sensitivity and capture viruses that are more difficult to culture, such as these EV-A viruses [31-33,36]. Another explanation could be a change in pathogenicity. We found a low level of circulation of EV-D68 (before 2010) and EV-C viruses, which is consistent with other studies (reviewed by [31]) and is suggested to be related to a low pathogenicity of these types [43]. Indeed, in 2010 and 2014, hospitals in Europe and the US reported increased detection of EV-D68 in respiratory samples from cases with severe respiratory disease [24-29,50,54-57].

The increase was related to genetic changes that could have driven a more severe pathogenicity rather than to changes in detection methods [50]. Unfortunately, clinical data were missing in the majority of cases of other viruses and more data are required to investigate whether pathogen drift could have additionally contributed to the increased detection of other NPEVS in certain years.

The data further suggest that the epidemic/endemic cyclic patterns might be driven by immunity; this could be due to antigenic diversification, waning immunity or simply lack of immunity. The frequency of infections at an extremely young age and at parenting age [58-60] suggests lack of protection by maternal antibodies [61]. Lack of immunity or waning immunity can be inversely related to the endemic cyclic patterns of the different types/strains; the types/strains that adults are exposed to are different from those circulating during their childhood when they frequently came in contact with EVs. An adult's immunity profile is thus directed against EV types not currently circulating, which has led to a high proportion of susceptible adults. In the case of antigenic diversification, the immunity that was predominantly built up in previous years may be lacking (loss of neutralisation capacity) or not effective against currently circulating antigenic variants (altered neutralisation capacity) [62]; this also leads to a high proportion of susceptible adults [24,50,54]. For EV-D68, the high proportion of infection among adult cases aged 45 to 65 years, and the divergence between the two lineages showing amino acid substitutions within the B/C loop, indeed suggest that antigenic diversification leading to altered neutralisation capacity plays a role in adult infections [50,54]. In contrast, E-30, CV-A9, E-18, CV-A6, CV-A16 and EV-A71, all frequently observed in adult infections, showed no antigenic diversification. However, we cannot rule out the occurrence of antigenic diversification among these types, as antigenic epitopes can also be found among other exposed VP1 loops and on other capsid proteins [44,51,52] not characterised by the Nix protocol.

Furthermore, no adults were identified among E-25, CV-B3 and E-9, strains proposed to have antigenic diversification. This would suggest that other factors act as a transmission bottleneck, such as differential receptor expression between adults and children. It has been suggested that viral characteristics such as receptor usage can account for the differential age of infection with several EV types and HPeV [19,63].

Another factor affecting the circulation patterns is seasonality. It has been suggested that infection frequency is dependent on the number of contacts or transmissions, which can be influenced by the season or the weather. The frequency of contacts is highest during the winter months [64-66], which could spur widespread transmission after an initial introduction and lead to unexplained illness outbreaks during winter season. The observed winter peak could be

related to the introduction of fast molecular methods that allowed rapid screening of samples throughout the years, revealing a more diverse seasonal pattern. EV should therefore also be considered in the differential diagnosis during winter seasons, contrary to the dogma describing EV infections as seasonal summer infections.

To investigate and understand the role of these factors (antigenic diversification, lack of or waning immunity, and seasonality) on the circulation pattern, full-length genetic and phenotypic analysis in combination with sero-population studies need to be conducted over an extended period. VIRO-TypeNed provides a platform to analyse these data in relation to one another.

Knowledge about the endemic/epidemic patterns can be used to investigate the possibility of type-specific vaccines [67]. Meanwhile, the data can be used to prescreen intravenous immunoglobulin (IVIG) products, where the knowledge of which types are currently circulating can enable a more effective use of IVIGs.

Furthermore, with the current developments in the antiviral field, EV infections may soon be classified as treatable rather than life-threatening [13]. The drugs currently in development show type-specific efficacy [68,69] and use requires the knowledge of which types are currently circulating or may cause an outbreak.

While VIRO-TypeNed provides data on NPEVs, the system also allows reporting the detection of PV. PV circulation might occur through inadvertent introduction of OPV, vaccine-derived PV (VDPV) or even wild-type PV, or via faecal excretions from migrants or travellers returning from endemic or OPV-using countries. Any type of PV isolation in the Netherlands leads to a public health alert because there is a large unvaccinated group (3% of the population) refusing vaccination for religious reasons that live in a closely isolated community (the Bible Belt). The 5'UTR PCR is able to detect all EVs including PV. Over the period studied, four OPV strains were reported. They had been detected by direct genotyping from clinical samples and had already been notified to the RIVM, and preventive actions for further spread had been taken. Given the very low circulation rate of PV in the Netherlands in non-epidemic years, EV-positive samples from which unique NPEV sequences are generated are considered PV-negative. However, as positive untyped samples can contain PV, laboratories are encouraged to send these samples, in particular those with suspicion of PV infection, to the RIVM for cultivation on L20B cells. As such, the surveillance capacity to exclude PV circulation in a molecular era is maintained.

Acknowledgements

We want to thank all the virologists participating in the Weekly Sentinel Surveillance System of the Dutch Working Group for Clinical Virology for collecting and providing positive samples for PV exclusion and NPEV typing. We would also like to thank Erwin Duizer for critically reading the manuscript and the laboratory staff of Laboratories participating in the VIRO-TypeNed project: Edin Jusic, and Jeroen Cremer (RIVM); Jolanda Kreeft-Voermans en Mark Verbeek (Erasmus MC); Lisette Rusman (LUMC); Hèlen van Raak en Judith Beuving (St Elisabeth Hospital); Gerrit Koen, Hetty van Eijk, Xiomara Thomas, Rene Minnaar, Karen Dijkamn-deHaan, Sjoerd Rebers (AMC); Randy Poelman, Coretta Van Leer-Buter, Renze Borger (UMCG).

Katja wolthers is partially funded by the Seventh Framework Programme of the European Union IAPP under contract PIAPP-GA-2013-612308 (AIROPico, www,airopico.eu).

Conflict of interest

None declared.

Authors' contributions

Kimberley Benschop performed the analyses of the data and wrote the first draft of the paper. Kimberley Benschop, Janette Rahamat-Langendoen Harrie van der Avoort, Eric. Claas, Suzan Pas, Rob Schuurman, Jaco Verweij, Katja Wolthers, and Hubert Niesters were responsible for collecting and recording the data in VIRO-TypeNed. Marion Koopmans critically reviewed the draft of the paper. All other authors reviewed the paper critically, and comments and suggestions were incorporated in the final version by Kimberley Benschop.

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Letter to the editor: The first tick-borne encephalitis case in the Netherlands: reflections and a note of caution

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Citation style for this article:
Clement J, Lagrou K, Saegeman V, Maes P, van Ranst M. Letter to the editor: The first tick-borne encephalitis case in the Netherlands: reflections and a note of caution. Euro Surveill. 2016;21(39):pii=30355. DOI: http://dx.doi.org/10.2807/1560-7917.ES.2016.21.39.30355

Article submitted on 30 August 2016 / accepted on 29 September 2016 / published on 29 September 2016

To the editor: We wish to offer some cautionary remarks concerning the report by de Graaf et al. [1] about the first human tick-borne encephalitis (TBE) infection in the Netherlands, acquired in June 2016 (and not in July, as incorrectly mentioned in the title). At first sight, this case, apparently proven by ELISA and confirmed by neutralisation tests (NT), seems extremely convincing, especially as it occurred after a bite from a local tick (species not mentioned) that was later found by qRT-PCR to be infected with a recently discovered Dutch TBE virus (TBEV).

However, this case could only be called 100% waterproof, if (i) the *Eurosurveillance* reader was given verifiable taxonomic data about the novel Salland TBEV and its relationship with other pathogenic TBEV and (ii) a convincing degree of homology was demonstrated between the TBEV isolated from the tick and the patient. Neither of these conditions was fulfilled in this Rapid communication. Admittedly, condition (ii), although an unquestionable paradigm for zoonotic infections, will be hard to fulfil in any forthcoming TBE case because TBEV is nearly always cleared from blood and cerebrospinal fluid (CSF) already at the start of the second TBE phase, i.e. before the patient is admitted with neurologic complications [2,3]. Consequently, and against our own expectations, RT-PCR has not become the ultimate tool for physicians attending a putative TBE case [4], as was again demonstrated in this case.

Thus, physicians have to rely solely on serological techniques, which have a number of flaws: TBEV shares common antigenic sites in its E protein with several other pathogenic flaviviruses, resulting in false-positive results in IgG and even IgM ELISA [2,4-7]. The ELISA seropositivity in the presented case could thus in theory be ascribed to the patient's yellow fever vaccination 11 years earlier [2,6,7]. Moreover, the specificity of gold standard NT, considered hitherto as sacrosanct, has recently also been called into question: in an animal study, four of five louping ill–infected sheep and two of 17 sera from West Nile virus (WNV)-infected horses, collected in TBE non-endemic regions and tested at British and German reference laboratories, reacted positive in TBE ELISA and even in TBE NT [7].

There are however still other, and more obvious, question marks concerning the evidence of true TBEV infection in the case under discussion. Firstly, two titres obtained in the NT remained unchanged (1/640). Although the crucial interval in days is not exactly specified (on days 24 and 36?), serological immobility is surprising for acute TBE when neutralising antibodies can be expected to increase. It is however compatible with a status of post-vaccination cross-protection. Secondly, and most importantly, CSF was only IgG-positive, while IgM was lacking. IgM-positivity in CSF is, however, paramount for diagnosing TBE and other flaviviral infections such as West Nile fever, to the extent that CSF IgG is not even considered, nor required for diagnosis, certainly not in a patient with prior contact with flaviviruses [2]. Moreover, IgM-positivity in CSF is almost invariably present by the 10th day of TBE illness [2] and peaking between day 9 and week 6 [3].

Finally, CSF findings are supportive of TBE diagnosis, when there is (i) pleocytosis with predominance of segmented granulocytes over lymphocytes, (ii) impairment of the blood–CSF barrier (increased CSF/serum albumin ratio), and (iii) intrathecal synthesis of immunoglobulins, predominantly of IgM [2]. None of these techniques were applied in the current case. As for the pleocytosis, an almost exclusive mononuclear cell reaction was found, which, to our knowledge, is highly unusual for TBE.

Since CSF, for unclear reasons, remained only IgG-positive, determining the CSF/serum IgG ratio could be helpful in proving or disproving the TBE origin of this diagnostically challenging case.

Conflict of interest

None declared.

Authors' contributions

Jan Clement conceived the idea of a Correspondence Letter, and wrote the first draft. Katrien Lagrou, Veroniek Saegeman and Piet Maes revised the manuscript. Veroniek Saegeman reviewed the recent TBE literature, and Marc Van Ranst revised and edited the final draft. All co-authors read and accepted this final draft.

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Author's reply: The first tick-borne encephalitis case in the Netherlands: reflections and a note of caution

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Citation style for this article:
Hira V, de Graaf JA, Rockx B, on behalf of the authors of the original article. Author's reply: The first tick-borne encephalitis case in the Netherlands: reflections and a note of caution. Euro Surveill. 2016;21(39):pii=30356. DOI: http://dx.doi.org/10.2807/1560-7917.ES.2016.21.39.30356

Article submitted on 20 September 2016 / accepted on 23 September 2016 / published on 29 September 2016

To the editor: We thank Clement et al. for their interest in our report on the first tick-borne encephalitis virus (TBEV) infection in the Netherlands (which was acquired in May, but diagnosed in July). They express their concerns about the correctness of the diagnosis and correctly point out several oddities and diagnostic challenges in the case. However, despite these uncertainties, we have no doubt that our patient was infected with TBEV because of several reasons.

Firstly, we did not rely solely on serological techniques, as the patient had provided us with the tick that had bitten him; the species was phenotypically not determinable because it had dried. The tick was TBEV-positive in qRT-PCR, not for the novel Dutch TBEV (a separate manuscript with sequence data of this virus is currently under review), but for a TBEV strain very closely related to the Neudörfl strain, implying the presence of several distinct strains of TBEV in the Netherlands. In our opinion, knowing that the patient had been bitten by a tick proven to be TBEV-infected, drastically increases the chance that compatible symptoms and positive anti-TBEV serology was attributable to a true TBEV infection.

Secondly, according to the paper by Holzmann and the recent European Union case definition for TBE, our patient met the criteria for a proven/confirmed TBEV infection. These criteria are, among others: symptoms of inflammation of the central nervous system and presence of specific IgM and IgG antibodies in blood and/or cerebrospinal fluid (CSF), in the absence of vaccination in the previous months [1,2]. In our case, the specificity of serum antibodies was confirmed with neutralisation tests (NT). Although, based on one study in animals [3], the specificity of NT is disputed, we believe the antibodies in our case were specific because NT titres were high. As the samples (indeed taken on days 24 and 36) were not diluted beyond 1/640 (the goal of the NT was to confirm the specificity of our ELISA results), we do not know the exact titres and thus their dynamics. In the cited animal study, cross-reactivity with louping ill virus was observed and all West Nile virus-infected horses were negative in TBEV NT [3]. As louping ill virus is not known to circulate in the Netherlands, we do not believe this has affected our results. Positive anti-TBEV antibodies may indeed have been due to a yellow fever vaccination 11 years earlier, but this is only true for IgG. IgM is only detectable for several months after vaccination [1]. Furthermore, the substantial decrease in anti-TBEV IgM concentration between days 24 and 36 cannot be explained by this vaccination, but is not unusual in a recent TBEV infection [1].

Thirdly, we should indeed have determined a CSF/serum IgG ratio. We recently determined this ratio and found it to be negative. Intrathecally produced anti-TBEV antibodies are, however, not mandatory for a definite diagnosis. Although the presence of intrathecally produced antibodies is indeed supportive of the diagnosis, they are not found in all cases. Kaise and Holzmann, for example, found that 16% of TBEV-infected patients did not have intrathecally produced anti-TBEV antibodies at hospital admission [4]. More recently, Henningsson et al. described a similar case, also without anti-TBEV antibodies in CSF [5].

In conclusion, we agree that our case had several unusual aspects, such as the predominant mononuclear cell reaction in CSF and the lack of intrathecally produced anti-TBEV antibodies. We believe, however, that our patient had a proven TBEV infection, based on the presence of compatible clinical symptoms, the presence of TBEV-specific antibodies in two different assays and the high levels of TBEV in the tick that had bitten the patient

Conflict of interest

None declared.

Authors' contributions

VH drafted the manuscript, BR and JAG critically revised the manuscript.

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Expert committee declares WHO Region of the Americas measles-free

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Citation style for this article:
Eurosurveillance editorial team. Expert committee declares WHO Region of the Americas measles-free. Euro Surveill. 2016;21(39):pii=. DOI: http://dx.doi.org/30360

Article published on 29 September 2016

The World Health Organization (WHO) Region of the Americas has been declared measles-free.

During a Pan American Health Organisation (PAHO)/WHO meeting on 27 September 2016 the International Expert Committee (IEC) for Documenting and Verifying Measles, Rubella, and Congenital Rubella Syndrome Elimination in the Americas announced that measles has been eliminated from the region. This follows a campaign which has lasted 22 years since efforts started in 1994, when the 35 countries of the Region launched an initiative to eliminate measles, rubella and congenital rubella syndrome.

The road to measles elimination has not been smooth. Measles transmission in the Region had been considered interrupted since 2002, when the last endemic case was reported in the Americas. Between 2003 and 2010, there was an average of 153 cases annually, either imported or linked to imported cases. However, large outbreaks between 2011 and 2015 in Brazil, Canada, Ecuador, and the United States resulted in 8–12 times more reported cases than in the preceding period. Brazil was the last country of the Region to experience a prolonged measles outbreak, from 2013 to 2015 [1]. In April 2015, the IEC declared that endemic measles transmission had emerged in Brazil. Brazil considered that measles transmission ended in July 2015, after the last confirmed case had been reported on 13 June 2013 [2]. The IEC reviewed evidence on measles elimination presented by all the countries of the Region between 2015 and August 2016 and decided that it met the established criteria for elimination. The process included six years of work with countries to document evidence of the elimination [1].

References

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‘European Antibiotics Awareness Day’ wins ‘European Health Award 2016’ accolade

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Citation style for this article:
Eurosurveillance editorial team. ‘European Antibiotics Awareness Day’ wins ‘European Health Award 2016’ accolade. Euro Surveill. 2016;21(39):pii=. DOI: http://dx.doi.org/30354

Article published on 29 September 2016

On 28 September 2016 the ‘European Antibiotic Awareness Day’ (EAAD), a health initiative coordinated by the European Centre for Disease Prevention and Control (ECDC) won the ‘European Health Award’, a prize awarded each year by the European Health Forum Gastein.

The EAAD, one of the six cross-border health projects short-listed for the European Health Award aims to provide a platform to support the prudent use of antibiotics. Each year across Europe, the EAAD is marked by national campaigns during the week of 18 November. The goal of the EAAD is to provide the participating countries with evidence-based tools and other support for their campaigns. In 2015, with the support of World Health Organisation Europe, 41 European countries organised activities to mark the initiative. The EAAD partners with World Antibiotic Awareness Week.

The European Health Award, established in 2007, aims to honour initiatives aiming to improve public health or healthcare in Europe.

Read more about EAAD here

Articles about EAAD in *Eurosurveillance*:

http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20928

http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19280